Factors Involved in the Differentiation of TGF-β-Producing Cells from Naive CD4$^+$ T Cells: IL-4 and IFN-γ Have Opposing Effects, While TGF-β Positively Regulates Its Own Production

Robert A. Seder,[1]* Thomas Marth,† Myra C. Sieve,* Warren Strober,† John J. Letterio,‡ Anita B. Roberts,‡ and Brian Kelsall†

TGF-β has been shown to play a central role in regulating inflammatory responses; thus, understanding the factors involved in the generation of TGF-β-producing cells could lead to interventions that are useful in effecting disease progression. In initial studies, the capacity of naive CD4$^+$ T cells from TCR transgenic (Tg) mice to produce TGF-β following primary and secondary stimulation was assessed. TGF-β, IL-4, or IFN-γ production could not be detected from highly purified naive CD4$^+$/lymphocyte endothelial cell adhesion molecule (LECAM)-1^{high} cells following primary stimulation for 36 h with plate-bound anti-CD3, anti-CD28, and IL-2. This population was subsequently used to study the differentiation of TGF-β-producing CD4$^+$ T cells. In further studies, naive CD4$^+$/LECAM-1^{high} cells from TCR transgenic mice of both the BALB/c and B10.A backgrounds were stimulated with T-depleted spleen cells (TDS) and specific peptide in the presence of various cytokines and/or cytokine antagonists for 5 days, restimulated, and TGF-β, IL-4, and IFN-γ production were measured. Priming conditions favoring high IL-4 production and/or low IFN-γ production greatly enhanced TGF-β production in secondary cultures. Furthermore, the presence of IL-10 in cultures was associated with an increase in TGF-β production following restimulation. The importance of IL-4 and IFN-γ in regulating TGF-β production was confirmed in studies showing that cells from IFN-γ$^{-/-}$ mice produced more TGF-β, while cells from IL-4$^{-/-}$ mice produced less TGF-β compared with wild-type controls. Finally, the addition of exogenous TGF-β to priming cultures significantly enhanced the production of TGF-β upon restimulation, demonstrating that TGF-β has a role in self-regulating its own production. *The Journal of Immunology,* **1998, 160: 5719–5728.**

Transforming growth factor-β is a largely inhibitory cytokine increasingly recognized as a key regulator of the inflammatory response occurring in various types of infectious and autoimmune diseases (1–3). In studies of experimental infection with *Leishmania* species (1) and *Toxoplasma gondii* (2), infections that require vigorous Th1 responses for effective immunity, more virulent disease occurs if infected animals are administered TGF-β, and less virulent disease occurs if animals are treated with anti-TGF-β; not surprisingly, these effects were due to the ability of TGF-β to inhibit Th1 responses. In addition, in experimental models of autoimmunity, such as experimental autoimmune encephalomyelitis (EAE) (4–6) and experimental colitis (7), TGF-β-producing cells inhibit the pathologic inflammatory response, while administration of anti-TGF-β either abrogates the protective effect of oral tolerization or exacerbates the underlying inflammation (5, 7). Taken together, these studies establish that TGF-β production is an important negative regulator of inflammatory response. It is thus of considerable interest to determine the conditions that affect the differentiation of TGF-β-producing cells.

While the factors controlling the development of Th1 and Th2 cells have been well defined (8–13), relatively little is known concerning the factors controlling the development of TGF-β-producing cells. On the one hand, there are several reports linking IL-4 production to such development (14–16), but this has so far consisted of studies of whole tissues, bulk cell populations, and cell lines, which show that IL-4- and TGF-β-producing cells develop under similar conditions. In addition, the requirement of IL-4 for TGF-β induction was questioned by the studies of Powrie et al. (17), who showed that resolution of a TGF-β-dependent experimental colitis occurs following adoptive transfer of CD4$^+$ T cells from IL-4$^{-/-}$ mice. In addition, it has been reported that TGF-β production could be enhanced by neutralization of IL-12 and IFN-γ (18) in the absence of an effect on IL-4 production, again indicating that the generation of TGF-β-producing cells occurs independently of the Th2 response. These observations indicate that the factors influencing TGF-β production are less well defined than those regulating IFN-γ and IL-4 production.

In the studies reported here, we have addressed this latter point by examining the conditions under which naive CD4$^+$ T cells differentiate into TGF-β-producing cells in vitro. We found that such differentiation occurs under priming conditions favoring induction of Th2 and inhibition of Th1 responses. This finding was corroborated by the fact that primed CD4$^+$ T cells from IL-4$^{-/-}$ mice produced less TGF-β, while cells from IFN-γ$^{-/-}$ mice produced more TGF-β, when compared with cells from wild-type mice. In

*Lymphokine Regulation Unit and †Mucosal Immunity Section, Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; and ‡Laboratory of Chemoprevention, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

Received for publication December 4, 1997. Accepted for publication February 6, 1998.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Address correspondence and reprint requests to Dr. Robert A. Seder, Clinical Immunology Section, Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, Building 10, Room 11C215, National Institutes of Health, Bethesda, MD 20892. E-mail address: rseder@nih.gov

0022-1767/98/$02.00

further studies, we showed that addition of IL-10 to primary cultures enhanced the amount of TGF-β and inhibited the amount of IFN-γ produced upon restimulation, consistent with the fact that inhibiting IFN-γ directly (as noted above) or indirectly (i.e., via IL-10) leads to increased TGF-β production.

Finally, we showed that TGF-β itself was able to strikingly enhance production of TGF-β following restimulation. This effect is both direct, occurring independently of IL-4 and/or IFN-γ, and indirect, occurring through the ability of TGF-β to inhibit IFN-γ produced in priming cultures.

Materials and Methods

Animals

TCR transgenic (Tg)[2] mice were produced as previously described (11). All mice used in experiments were heterozygous for the integration of TCR variable region Vα11 and Vβ3 chains and maintained by backcrossing to B10.A mice. Homozygous 6- to 12-week-old female OVA 323–339-specific and I-A^d-restricted DO11.10TCR-αβ Tg mice (19) on a BALB/c genetic background were kindly provided by Dr. Dennis Loh (Washington University, St. Louis, MO). IL-4-deficient mice derived on a BALB/c background were obtained from Nancy Noben-Trauth (NIAID, Bethesda, MD) (20). IFN-γ-deficient mice derived on a C57BL/6 background (21) were obtained from Genentech (San Francisco, CA). Virus-free B10.A and BALB/c female mice (6–12 wk old) were obtained from the Division of Cancer Treatment, National Cancer Institute (Frederick, MD).

Tissue culture medium

RPMI 1640 medium supplemented with 10% heat-inactivated FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), 1 mM sodium pyruvate, L-glutamine (2 mM), and 2-ME (50 μM) was used for all primary stimulations. For all secondary stimulations, cells were cultured in serum-free medium in which Nutridoma SP was substituted for FCS (Boehringer Mannheim, Indianapolis, IN).

Peptide

Peptides with the sequences corresponding to residues 88–104 of pigeon cytochrome *c* (KAERADLIAYLKQATAK) and to residues 323–339 (ISQAVHAAHAEINEAGR) of OVA were synthesized by the National Institute of Allergy and Infectious (NIAID) Diseases Laboratory of Molecular Structure.

Recombinant lymphokines

Recombinant mouse IL-2 and IL-10 were purchased from Genzyme (Cambridge, MA). Mouse rIL-4 was obtained by a baculovirus expression system utilizing a vector into which the IL-4 gene had been inserted by C. Watson (Laboratory of Immunology, NIAID). One unit of IL-4 is equivalent to ~0.5 pg. Mouse rIL-12 was a generous gift of Genetics Institute (Cambridge, MA). Human TGF-β was purchased from R&D Systems (Minneapolis, MN).

Antibodies

Purified monoclonal rat anti-mouse IL-4 (11B11) (22) was prepared by Verax Corporation (Hanover, NH). Rat anti-mouse IFN-γ (XMG 1.2) (23), lymphocyte endothelial cell adhesion molecule (LECAM)-1 (MEL14) (24), was purchased from PharMingen (San Diego, CA). Sheep anti-IL-12 was a generous gift of Genetics Institute. Chicken anti-TGF-β was purchased from R&D Systems. Anti-CD28 ascites was a generous gift of Dr. James Allison (University of California, Berkeley, CA). Anti-CD3 (2C11) (25) was a generous gift of Dr. Jeffrey Bluestone (University of Chicago, Chicago, IL).

Preparation of accessory cells

TDSs were prepared from B10.A mice or BALB/c mice as previously described (10) and used as APCs. There were no T cells noted in the TDSs as assessed by FACS.

Preparation of T cells

$CD4^+$ T cells from transgenic mice were prepared in the following manner. Pooled lymph node cells and spleen cells were removed from TCR/Tg, IL-4, or IFN-$\gamma^{-/-}$ mice (4–8 wk of age) and passed over a negatively selecting CD4 subset enrichment column (R&D Systems). Cells were then stained with phycoerythrin (PE)-labeled anti-CD4 and FITC LECAM-1. These cells were subjected to FACS with a FACStar^{Plus} (Becton Dickinson, Sunnyvale, CA). Postsort analysis revealed >99.6% $CD4^+$/LECAM-1^{high} cells.

Primary and secondary stimulation of Tg T cells

For primary stimulation, $CD4^+$/LECAM-1^{high} and $CD4^+$/LECAM-1^{low} T cells obtained by FACS were stimulated on plates coated with anti-CD3 (10 μg/ml; PB-anti-CD3) plus soluble anti-CD28 (1:500 serial dilution of ascites) and IL-2 (10 U/ml) for 36 to 48 h in the presence of additional cytokines or cytokine antagonists. TGF-β, IL-4, and IFN-γ production were assessed in culture supernatants by cytokine-specific ELISA.

For secondary stimulation, priming cultures were first established by adding 3 to 5 × 10^5 sorted $CD4^+$/LECAM^{high} T cells to individual wells of 24-well plates in a total volume of 1.5 ml of media with irradiated (1000 rad) TDS cells, Ag (peptide or soluble anti-CD3), and various cytokines and anti-cytokines, as described in *Results*. After 48 h, cells were transferred to 6-well plates and supplemented with 3 ml of fresh medium only for an additional 2 to 3 days to allow for further expansion. $CD4^+$ T cells were then washed three times, and 1 to 2 × 10^5 cells in a total volume of 200 μl were restimulated on 96-well microtiter plates coated with anti-CD3 (PB-anti-CD3) in the presence of soluble anti-CD28 (1/500 final dilution) and IL-2 (10 U/ml). Thirty-six hours later, supernatants were collected and assayed for lymphokine. All secondary restimulations were done in serum-free medium in which Nutridoma SP was substituted for FCS. It should be noted that, at the time of restimulation, cells were >99% $CD4^+$ T cells.

Measurement of lymphokine production

IFN-γ was assayed by a specific two-site ELISA with reference standard curves using known amounts of recombinant IFN-γ. The lower limit of detection was 0.3 ng/ml. A cytokine-specific ELISA kit for IL-4 was purchased from Endogen (Boston, MA). The lower limit of sensitivity for the IL-4 ELISA was 3 U/ml. TGF-β1 and IL-10 levels were determined by ELISA from kits purchased from Genzyme. The lower limit of detection was 50 to 100 pg/ml for TGF-β1 and 30 to 60 pg/ml for IL-10. All measurements of TGF-β were done after supernatants were acidified as per the instructions by the manufacturer. All TGF-β detected was latent, since there was no TGF-β detected if the supernatants were not acidified. In all experiments, serial dilutions of supernatants were used to measure cytokine content for IL-4, IFN-γ, and TGF-β to ensure that all values were obtained from the linear portion of the standard curve. In addition, IL-4, IFN-γ, and TGF-β production were all assessed from the same supernatants. Supernatants were assayed in triplicate for all experiments. The SEM was <10% in all experiments.

Statistical analysis

Normally distributed continuous variable comparisons were done employing the Student *t* test.

Results

Following primary stimulation, $CD4^+$/LECAM-1^{high} (naive) T cells do not produce TGF-β, while $CD4^+$/LECAM-1^{low} (memory) T cells produce small amounts of TGF-β

In our initial studies, we determined the TGF-β production from naive and memory $CD4^+$ T cells after primary stimulation in vitro in the absence of accessory cells. Accordingly, $CD4^+$/LECAM-1^{high} and $CD4^+$/LECAM-1^{low} T cells representing naive and memory cells, respectively, were obtained by cell sorting from DO11.10 OVA-TCR/Tg mice (BALB/c background) and stimulated on plates coated with anti-CD3 in the presence of soluble anti-CD28 and IL-2. After 36 h, culture supernatants were harvested and assayed for secreted TGF-β.

As shown in Table I, $CD4^+$/LECAM-1^{high} (naive) T cells in primary cultures produced low to undetectable amounts of TGF-β as well as low to undetectable amounts of IFN-γ or IL-4. In contrast, $CD4^+$/LECAM-1^{low} (memory) T cells stimulated in a similar fashion produced low but detectable amounts of TGF-β (205 pg/

[2] Abbreviations used in this paper: Tg, transgenic; TDS, T-depleted spleen cell; LECAM, lymphocyte endothelial cell adhesion molecule; OVAp, OVA-peptide; CYTO-C, cytochrome *c*.

Table I. *Production of TGF-β, IFN-γ, and IL-4 following primary stimulation*[a]

	TGF-β (pg/ml)		IFN-γ (ng/ml)		IL-4 (U/ml)	
	$CD4^+$/LECAM-1^{high}	$CD4^+$/LECAM-1^{low}	$CD4^+$/LECAM-1^{high}	$CD4^+$/LECAM-1^{low}	$CD4^+$/LECAM-1^{high}	$CD4^+$/LECAM-1^{low}
Expt. 1						
$CD4^+$ T cells + PB-anti-CD3 + anti-CD28 + IL-2	<100	205	<0.3	1.6	40	28,696
+IL-4	215	244	<0.3	1.7	(620)	(31,564)
+anti-IFN-γ	<100	293	(<0.3)	(<0.3)	78	21,232
+anti-IL-4	<100	156	<0.3	1.9	(46)	(5,864)
Expt. 2						
$CD4^+$ T cells + TDS + OVAp	<100	329	<0.3	2.2	<3	5,036
$CD4^+$ T cells + PB-anti-CD3 + anti-CD28 + IL-2	<100	254	<0.3	3.1	150	5,706
+IL-4	112	253	<0.3	3.5	(1,400)	(6,092)
+anti-IL-4	<100	190	<0.3	3.5	(<3)	(216)

[a] $CD4^+$/LECAM-1^{high} and $CD4^+$/LECAM-1^{low} T cells obtained by FACS were stimulated on plates coated with anti-CD3 $(PB\text{-}anti\text{-}CD3)^+$ soluble anti-$CD28^+$ IL-2 for 48 h in the presence of IL-4 (1000 U/ml), anti-IFN-γ (20 μg/ml), or anti-IL-4 (20 μg/ml). TGF-β, IL-4, and IFN-γ production were assessed by cytokine-specific ELISA. The lower limit of detection for TGF-β was <100 pg/ml; for IL-4, <3 U/ml; for IFN-γ, <0.3 ng/ml. Values represent the means of triplicate cultures. In groups in which IL-4 or anti-IL-4 was added, the values for IL-4 are listed in parentheses due to the possibility that these factors may affect the results of the ELISA. The SEM was <10% for all cytokines. These two experiments are representative of three additional experiments.

ml) as well as substantial amounts of IL-4 along with low amounts of IFN-γ. To determine whether these levels of TGF-β production could be influenced by the presence or absence of other cytokines in the cultures, we also stimulated cells in the presence of added cytokines and anti-cytokine Abs. As also shown in Table I, we found that the addition of IL-4, but not anti-IL-4 or anti-IFN-γ, induced the production of small but detectable amounts of TGF-β (215 pg/ml) in cultures of naive (LECAM-1^{high}) T cells, but none of these additions appreciably altered TGF-β production in cultures of memory (LECAM-1^{low}) T cells.

In additional studies, the capacity of naive and memory T cells to produce TGF-β when stimulated with APCs (irradiated TDSs) and OVA-peptide (OVAp), rather than anti-CD3, anti-CD28, and IL-2, was determined. As shown in the second experiment in Table I, naive T cells stimulated with OVAp and TDS still do not produce detectable TGF-β, whereas memory $CD4^+$ T cells produced both TGF-β and IL-4. Overall, these studies show that memory $CD4^+$ T cells produce low amounts of TGF-β in association with the production of large amounts of IL-4 and minimal amounts of IFN-γ, while naive $CD4^+$ T cells produce little or no TGF-β upon primary stimulation in vitro. Thus, naive $CD4^+$/LECAM-1^{high} cells constitute a defined population of cells that is suitable for the study of the differentiation of cells capable of producing TGF-β following in vitro priming.

Priming of naive $CD4^+$/LECAM-1^{high} T cells (derived from either BALB/c or B10.A mice) for TGF-β production is enhanced by the presence of IL-4 and absence of IFN-γ in the priming cultures

In light of the above studies, we next determined the ability of $CD4^+$/LECAM-1^{high} (naive) T cells from TCR/Tg mice to undergo differentiation into TGF-β-producing T cells when primed in vitro under various conditions in secondary cultures. In these studies, we utilized $CD4^+$ T cells from OVA-TCR/Tg mice derived on a BALB/c background (as in the studies above) as well as $CD4^+$ T cells from cytochrome *c* (CYTO-C)-TCR/Tg mice derived on a B10.A background, recognizing that mice of these respective backgrounds produce different levels of IL-4 and IFN-γ under "neutral" baseline priming conditions (i.e., in the absence of exogenous cytokines) that could affect the generation of TGF-β-producing cells (12). Accordingly, naive $CD4^+$/LECAM-1^{high} cells from OVA-TCR/Tg mice and CYTO-C-TCR/Tg mice were stimulated in priming cultures for 5 days with TDS plus OVAp or CYTO-C peptide, respectively, then extensively washed and restimulated in secondary cultures with immobilized anti-CD3 plus soluble anti-CD28 and IL-2 for 2 days, after which cell culture supernatants were harvested and assayed for cytokines. As shown in Table II, depicting the mean values obtained from three independent studies, naive $CD4^+$ T cells from OVA-TCR/Tg mice primed under these baseline conditions produced much higher levels of TGF-β following secondary stimulation than similarly primed T cells from CYTO-C-TCR/Tg mice. In the studies of OVA-TCR/Tg mice (Table II, Expt.1), the high baseline production of TGF-β by naive T cells was enhanced by the addition of IL-4 or anti-IL-12 plus anti-IFN-γ to the priming cultures ($p < 0.05$), whereas addition of anti-IL-4 diminished TGF-β production ($p < 0.05$). Furthermore, in the studies of naive T cells from CYTO-C-TCR/Tg mice, the low level of baseline production of TGF-β was enhanced by the addition of IL-4, either in the presence ($p < 0.05$) or absence ($p < 0.05$) of anti-IL-12 plus anti-IFN-γ in the priming cultures. Addition of anti-IL-12 plus anti-IFN-γ alone to the priming cultures also led to an increase in TGF-β production compared with what was produced under baseline priming conditions. These studies show that the baseline generation of TGF-β-producing cells is influenced by the strain of origin of the naive $CD4^+$ T cells. In addition, they show that regardless of baseline TGF-β production, such generation is positively regulated by priming in the presence of IL-4 and is negatively regulated by priming in the presence of IL-12 and IFN-γ. It should be noted that, while it is likely that the ability of IL-12 to down-regulate TGF-β is mediated through IFN-γ, a direct role for IL-12 cannot be excluded (10).

The results shown above also suggest that conditions leading to the differentiation of naive $CD4^+$ T cells into TGF-β-producing cells would also result in the induction of IL-4-producing cells but not to the induction of IFN-γ-producing cells. To investigate this possibility, we measured IL-4 and IFN-γ production from the same cultures used to assess TGF-β production to see whether there was a positive correlation between TGF-β and IL-4 production and a negative correlation between TGF-β and IFN-γ production. As

Table II. *Priming for TGF-β production in secondary cultures is correlated with high production of IL-4 and low production of IFN-γ from naive CD4+ T cells isolated from OVA-TCR/Tg or cytochrome c TCR/Tg mice*[a]

Priming Conditions	Secondary Stimulation Conditions: CD4+ T Cells + PB-anti-CD3 + anti-CD28 + IL-2		
	TGF-β (pg/ml)	IFN-γ (ng/ml)	IL-4 (U/ml)
Expt. 1: OVA-TCR/Tg mice (BALB/c)			
Sorted CD4+/LECAM-1^{high} T cells + TDS + OVAp + IL-2	499 ± 156	11.8 ± 1.24	12,916 ± 16,968
+IL-4	924 ± 62[b]	<0.3	59,216 ± 39,281
+anti-IL-4	270 ± 80[b]	13.3 ± 8.6	451 ± 320
+anti-IFN-γ + anti-IL-12	872 ± 49[b]	<0.3	23,721 ± 13,473
Expt. 2: CYTO-C-TCR/Tg mice (B10.A)			
Sorted CD4+/LECAM-1^{high} T cells + TDS + CYTO-Cp + IL-2	191 ± 60	0.8 ± 0.6	25 ± 35
+IL-4	704 ± 127[b]	<0.3	24,445 ± 17,901
+anti-IL-4	231 ± 93	2.5 ± 0.8	20 ± 1
+anti-IFN-γ + anti-IL-12	379 ± 56	<0.3	911 ± 1,256
+anti-IFN-γ + anti-IL-12 + IL-4	797 ± 167[b]	<0.3	3,945 ± 1,071

[a] Sorted CD4+/LECAM-1^{high} transgenic T cells (5×10^5) were stimulated for 4 days with TDS (3×10^6), OVA, or CYTO-C peptide (1 μM), IL-2 (10 U/ml), with IL-4 (1000 U/ml) in the presence or absence of anti-IFN-γ plus anti-IL-12 (20 μg/ml) or anti-IL-4 (20 μg/ml). Cells were restimulated and assessed for production of TGF-β, IL-4, and IFN-γ as outlined in Table I. Results from cells obtained from OVA-TCR/Tg and CYTO-C-TCR/Tg are pooled from three separate experiments.
[b] $p < 0.05$ when compared with priming under neutral conditions. Statistical analysis was performed by Student's *t* test.

shown in Table II (Expt. 1), appreciable amounts of IL-4 and IFN-γ are present in supernatants of secondary cultures of cells primed under baseline conditions. Furthermore, addition of IL-4 or anti-IL-12 plus anti-IFN-γ to priming cultures led to an increase in both TGF-β and IL-4 production and markedly reduced IFN-γ production. By contrast, addition of anti-IL-4 to the priming cultures led to a marked reduction in IL-4 production as well as a decrease in TGF-β production. Overall, these results show that increased TGF-β production occurs under conditions that favor high IL-4 and low IFN-γ production in secondary cultures.

Finally, as shown in Table I, TGF-β production by CD4+/LECAM-1^{low} (memory) T cells stimulated with anti-CD3 plus CD28 plus IL-2 was not appreciably affected by addition of cytokines or cytokine antagonists to the cultures. This result suggests that TGF-β production from previously activated cells may be relatively unaffected by changes in the cytokine environment. To address this issue more directly, CD4+/LECAM-1^{high} T cells were stimulated under baseline priming conditions and then restimulated in secondary cultures in the presence or absence of cytokine antagonists, after which TGF-β production was measured. Cells stimulated under baseline conditions as described above and restimulated with anti-CD3 plus CD28 plus IL-2 produced 801 pg/ml of TGF-β. TGF-β production following secondary stimulation in the presence of anti-IFN-γ or anti-IL-4 was 857 pg/ml and 771 pg/ml, respectively. Thus, it is apparent that, once cells become committed to making TGF-β in priming cultures, they are relatively resistant to further regulation by IL-4 or IFN-γ, at least over the 36-h time period of the restimulation culture.

IL-10 enhances priming of CD4+ T cells for TGF-β production

Based on the observation that IL-10 is a potent inhibitor of IFN-γ and IL-12 production, it was of interest to determine what effect IL-10 would have on the induction of TGF-β-producing cells. As shown in Table IIIA, in data from two independent experiments, the presence of IL-10 in primary cultures led to a twofold increase in TGF-β production in secondary cultures ($p < 0.02$). This was associated with a decrease in IFN-γ, suggesting that the presence

Table III. *TGF-β production from primed CD4+ T cells is enhanced by the presence of IL-10 in primary cultures*

Priming Conditions	Secondary Stimulation Conditions: CD4+ T Cells + PB-anti-CD3 + anti-CD28 + IL-2			
	TGF-β (pg/ml)	IFN-γ (ng/ml)	IL-4 (U/ml)	IL-10 (pg/ml)
A. CD4+/LECAM-1^{high} T cells + TDS + OVAp + IL-2[a]	520 ± 86.7[b]	7.5 ± 1.05	2,294 ± 1,285	
+IL-10	959 ± 181[b]	1.68 ± 0.49	49,897 ± 25,025	
+IL-10 + anti-IL-4	778 ± 59	5.9 ± 1.82	4,044 ± 2,021	
+anti-IL-4	404 ± 67	11.8 ± 1.94	141 ± 116	
B. CD4+/LECAM-1^{high} T cells + TDS + OVAp + IL-2[c]	279			200
+IL-10	775			610
+IL-10 + anti-IL-4	852			126
+IL-4	1,309			>5,400

[a] In data combined from two independent experiments, sorted CD4+/LECAM-1^{high} transgenic T cells (5×10^5) from OVA-TCR/Tg were primed by stimulation for 4 days with TDSs (3×10^6), peptide (1 μM), or IL-2 (10 U/ml) in the presence of IL-10 (10 ng/ml) and/or anti-IL-4 (20 μg/ml). Cells were washed extensively, recultured in serum-free media, and restimulated at 2×10^5 cells/200 μl on plates coated with anti-CD3 (PB-anti-CD3) plus soluble anti-CD28 plus IL-2 for 36 h. Supernatants were harvested, and TGF-β, IL-4, and IFN-γ content were measured by ELISA.
[b] $p < 0.02$ represents a statistical difference as assessed by Student's *t* test in comparing TGF-β produced under neutral conditions with that produced in the presence of IL-10.
[c] In a third experiment, cells were primed in a similar manner as *A*. Supernatants were harvested, and TGF-β and IL-10 content were measured by ELISA. SEM for both cytokines was <10%.

Table IV. *$CD4^+$ cells from IFN-$\gamma^{-/-}$ mice have an increased capacity to produce TGF-β following in vitro priming*[a]

Priming Conditions	Secondary Stimulation Conditions: $CD4^+$ T Cells + PB-anti-CD3 + anti-CD28 + IL-2		
	TGF-β (pg/ml)	IFN-γ (ng/ml)	IL-4 (U/ml)
Sorted $CD4^+$/LECAM-1^{high} T cells from IFN-$\gamma^{-/-}$ mice			
$CD4^+$ T cells + TDS + anti-CD3 + IL-2	513 ± 214	<0.3	44,401 ± 2,938
+IL-4	787 ± 243	<0.3	53,770 ± 10,992
Sorted $CD4^+$/LECAM-1^{high} T cells from IFN-$\gamma^{+/+}$ mice			
$CD4^+$ T cells + TDS + anti-CD3 + IL-2	359 ± 167	2.4 ± 0.69	36,462 ± 15,841
+anti-IFN-γ + anti-IL-12	652 ± 96	<0.3	77,285 ± 151
Total/unsorted $CD4^+$ cells from IFN-$\gamma^{-/-}$ mice			
$CD4^+$ T cells + TDS + anti-CD3 + IL-2	978 ± 23[b]	<0.3	29,194 ± 764
+anti-IL-4	685 ± 9.4	<0.3	2,055 ± 458
Total/unsorted $CD4^+$ cells from IFN-$\gamma^{+/+}$ mice			
$CD4^+$ T cells + TDS + anti-CD3 + IL-2	312 ± 110[b]	51.4 ± 22.6	11,701 ± 3,416
+anti-IFN-γ + anti-IL-12	820 ± 36	6.7 ± 2.32	49,819 ± 19,833

[a] In results combined from three independent experiments, total/unsorted CD4 and sorted $CD4^+$/LECAM-1^{high} T cells were isolated from IFN-$\gamma^{-/-}$ and IFN-$\gamma^{+/+}$ mice derived on a C57BL/6 background. In these experiments, $CD4^+$ T cells were approximately 95% pure by FACS analysis (70–75% $CD4^+$/LECAM^{high}, 20% $CD4^+$/LECAM^{low}) for both IFN-$\gamma^{-/-}$ and IFN-$\gamma^{+/+}$ mice (data not shown). Sorted $CD4^+$/LECAM-1^{high} or total/unsorted $CD4^+$ T cells (5×10^5) were stimulated for 4 days with TDSs (3×10^6) from IFN-$\gamma^{-/-}$ mice, soluble anti-CD3 (3 μg/ml), IL-2 (10 U/ml), in the presence of IL-4 (1000 U/ml), anti-IFN-γ anti-IL-12 (20 μg/ml), or anti-IL-4 (20 μg/ml). Cells were restimulated and assessed for production of TGF-β, IL-4, and IFN-γ as outlined in Table II.

[b] $p < 0.02$ represents a statistical difference as assessed by Student's t test in comparing TGF-β produced from total/unsorted $CD4^+$ T cells from IFN-$\gamma^{-/-}$ and IFN-$\gamma^{+/+}$ mice.

of IL-10 may exert a positive but indirect influence on the induction of TGF-β-producing cells through its inhibition of IFN-γ. It should be noted that, while IL-4 production was also increased by the presence of IL-10, this did not appear to contribute to the increase in TGF-β production, since the addition of anti-IL-4 did not substantially diminish production of TGF-β.

It has recently been demonstrated that the presence of IL-4 or IL-10 in priming cultures leads to the outgrowth of cell lines capable of producing IL-10 and, in some cases, TGF-β as well (26). This led us to study the role of IL-4 and IL-10 in regulating priming for IL-10 production so as to determine whether there was a correlation between induction of IL-10 and TGF-β. In an experiment addressing this point (Table III*B*), addition of IL-10 to priming cultures in the presence or absence of anti-IL-4 led to a threefold increase in production of TGF-β following restimulation, consistent with what was seen above. In addition, the presence of IL-10 also led to a threefold increase in IL-10 production that was abrogated by addition of anti-IL-4, suggesting that IL-4 had a role in the induction of IL-10-producing cells. This was supported by the finding that addition of IL-4 to priming cultures led to a striking increase in the production of IL-10 following restimulation. These results show that optimal priming for IL-10 and TGF-β are somewhat different. While the IL-10 effect of TGF-β priming is independent of IL-4, the IL-10 effect of IL-10 priming appears dependent on IL-4.

The ability of IL-4 to enhance both TGF-β and IL-10 raises the question of whether IL-4 is enhancing TGF-β indirectly through IL-10. To address this possibility, naive $CD4^+$/LECAM-1^{high} T cells from IL-10-deficient mice and control wild-type mice were primed under neutral conditions or in the presence of IL-4, and TGF-β production was assessed following restimulation. $CD4^+$ T cells from IL-$10^{-/-}$ mice stimulated under neutral conditions produced 245 pg/ml of TGF-β compared with 600 pg/ml from control mice, demonstrating that IL-$10^{-/-}$ mice make relatively less TGF-β than do control mice. Furthermore, addition of IL-4 to cultures of IL-$10^{-/-}$ mice increased TGF-β production from 245 pg/ml to 635 pg/ml, providing strong evidence that IL-4 can induce TGF-β in the complete absence of IL-10.

$CD4^+$ T cells from IFN-$\gamma^{-/-}$ mice have an increased capacity to produce TGF-β

Since IFN-γ appears to inhibit TGF-β priming, we next wished to determine whether $CD4^+$ T cells from IFN-$\gamma^{-/-}$ mice were biased toward increased TGF-β production. In these studies, $CD4^+$/LECAM-1^{high} (naive) T cells from IFN-$\gamma^{-/-}$ and IFN-$\gamma^{+/+}$ mice were stimulated in priming cultures under baseline conditions in the presence or absence of cytokines and/or cytokine antagonists and were then evaluated for their production of TGF-β, IL-4, and IFN-γ following restimulation under baseline conditions in secondary culture. As shown in Table IV, in results combined from three independent experiments, TGF-β production by cells from IFN-$\gamma^{-/-}$ mice primed under baseline conditions was modestly increased over that of similarly primed cells from IFN-$\gamma^{+/+}$ mice. Furthermore, the addition of IL-4 to priming cultures of cells from IFN-$\gamma^{-/-}$ mice resulted in a modest increase in both TGF-β and IL-4 production. Finally, addition of anti-IFN-γ plus anti-IL-12 to priming cultures containing naive cells from IFN-$\gamma^{+/+}$ mice resulted in an increase in TGF-β production similar to the amount detected with IFN-$\gamma^{-/-}$ mice primed under neutral conditions. These data are thus entirely consistent with the effects of IFN-γ and IL-4 on priming for TGF-β obtained in normal mice. In addition, they emphasize the fact that naive $CD4^+$ T cells from IFN-$\gamma^{-/-}$ mice can be positively primed by IL-4 to produce increased amounts of TGF-β, providing evidence that IL-4 has a direct positive effect (i.e., IFN-γ independent) on the differentiation of TGF-β-producing cells.

In additional experiments, we studied TGF-β, IL-4, and IFN-γ production from total/unsorted $CD4^+$ T cells from IFN-$\gamma^{-/-}$ and IFN-$\gamma^{+/+}$ mice following in vitro priming and restimulation, since this cell population contains a minor population of LECAM^{low} (memory) $CD4^+$ T cells that reflect in vivo priming and could thus influence the in vitro priming of the majority population of LECAM-1^{high} (naive) $CD4^+$ T cells. It should be noted that, in these experiments, comparison between total/unsorted $CD4^+$ T cells from IFN-$\gamma^{-/-}$ and IFN-$\gamma^{+/+}$ (wild-type) mice are valid, since these populations contained similar numbers of naive and

Table V. *CD4+ T cells from IL-4−/− mice have decreased capacity to produce TGF-β after priming*[a]

Priming Conditions	Secondary Stimulation Conditions: CD4+ T Cells + PB-anti-CD3 + anti-CD28 + IL-2		
	TGF-β (pg/ml)	IFN-γ (ng/ml)	IL-4 (U/ml)
Expt. 1			
Sorted CD4+/LECAM-1high T cells from IL-4−/− mice			
CD4+ T cells + TDS + anti-CD3 + IL-2	243 ± 140[b]	8.2 ± 4.7	<10
+anti-IFN-γ + anti-IL-12	344 ± 199	2.8 ± 1.6	<10
+IL-4	500 ± 288	2.2 ± 1.3	<10
Sorted CD4+/LECAM-1high T cells from IL-4+/+ mice			
CD4+ T cells + TDS + anti-CD3 + IL-2	679 ± 392[b]	1.97 ± 1.1	16,358 ± 9,444
+anti-IL-4	509 ± 294	9.76 ± 5.6	832 ± 480
Expt. 2			
Total/unsorted CD4+ T cells from IL-4−/− mice			
CD4+ T cells + TDS + anti-CD3 + IL-2	351	54.0	<10
+anti-IFN-γ + anti-IL-12	544	<0.3	<10
Total/unsorted CD4+ T cells from IL-4+/+ mice			
CD4+ T cells + TDS + anti-CD3 + IL-2	916	<0.3	63,692
Anti-IL-4	ND	ND	ND
Expt. 3			
Total/unsorted CD4+ T cells from IL-4−/− mice			
CD4+ T cells + TDS + anti-CD3 + IL-2	411	29.8	<10
+anti-IFN-γ + anti-IL-12	660	5.9	<10
Total/unsorted CD4+ T cells from IL-4+/+ mice			
CD4+ T cells + TDS + anti-CD3 + IL-2	1,509	3.6	31,906
+anti-IL-4	402	33.3	1,602

[a] Total/unsorted CD4 and sorted CD4+/LECAM-1high T cells were isolated from IL-4−/− and IL-4+/+ mice derived on a BALB/c background. Sorted CD4+/LECAM-1high (experiment No. 1) or total/unsorted CD4+ T cells (5×10^5) (experiments No. 2 and No. 3) were stimulated for 4 days with TDS (3×10^6) from IL-4−/− mice, soluble anti-CD3 (3 μg/ml), or IL-2 (10 U/ml) in the presence of IL-4 (1000 U/ml), anti-IFN-γ+ anti-IL-12 (20 μg/ml), or anti-IL-4 (20 μg/ml). Cells were restimulated and assessed for production of TGF-β, IL-4, and IFN-γ as outlined in Table III. In experiment No. 1, results are combined from three independent experiments.

[b] $p < 0.05$ represents a statistical difference in comparing the amount of TGF-β produced by IL-4−/− and IL-4+/+ CD4+ T cells.

memory cells (see Table IV legend). As shown in Table IV, total/unsorted CD4+ T cells from IFN-γ−/− mice primed under baseline conditions produced threefold more TGF-β ($p < 0.002$) and two- to fourfold more IL-4 compared with similarly primed cells from the IFN-γ+/+ mice. Furthermore, addition of anti-IL-4 to the priming cultures led to decreases in both TGF-β and IL-4 production. The fact that neutralization of IL-4 in priming cultures using total/unsorted CD4+ T cells from IFN-γ−/− mice diminishes both IL-4 and TGF-β provides additional evidence that IL-4 has a direct positive (IFN-γ-independent) effect on the generation of TGF-β-producing T cells.

IL-4 is not essential for TGF-β priming

The data presented so far suggest an important role for IL-4 in the generation of cells producing TGF-β, either directly or via an influence on IFN-γ production. To further address the role of IL-4 in TGF-β production, we performed studies similar to those described above with IFN-γ−/− mice with both CD4+/LECAM-1high (naive) T cells and total/unsorted CD4+ T cells derived from IL-4−/− and IL-4+/+ mice (both on a BALB/c background). As shown in Table V, in results combined from three independent experiments, naive T cells from IL-4−/− mice primed under baseline conditions and then evaluated for cytokine production after secondary stimulation produced two- to threefold less TGF-β than similarly primed cells from IL-4+/+ mice ($p < 0.02$). This increased production of TGF-β from cells of IL-4+/+ mice occurred concomitantly with high production of IL-4 but little IFN-γ in the secondary culture. Furthermore, addition of IL-4 to the priming cultures led to a two- to threefold increase in TGF-β by cells of IL-4−/− mice, while addition of anti-IFN-γ plus anti-IL-12 to the priming cultures was associated with only a modest increase in TGF-β production by cells from these mice.

As also shown in Table V (Expts.2 and 3), total/unsorted T cells from IL-4−/− mice primed under baseline conditions and then evaluated for cytokine production after secondary stimulation produced three- to fourfold less TGF-β than cells from IL-4+/+ mice primed in a similar manner. This observation, taken in conjunction with the observation that the addition of anti-IL-4 to priming cultures of cells from IL-4+/+ mice greatly diminished TGF-β production in secondary cultures (Expt.3), is consistent with the view that IL-4—while not essential—may be important for optimal generation of TGF-β-producing cells. Finally, addition of anti-IFN-γ plus anti-IL-12 to the priming cultures of total/unsorted cells from IL-4−/− mice led to a 50% increase in the production of TGF-β in both experiments, consistent with IFN-γ having a negative influence on TGF-β induction. These data thus provide further support for the idea that IFN-γ has a direct negative (IL-4-independent) effect in the generation of TGF-β-producing cells.

While the above results clearly show that cells producing TGF-β can differentiate in the absence of IL-4, they do not rule out the possibility that other Th2-type cytokines such as IL-13 operate in the absence of IL-4 to influence TGF-β induction. To address this possibility, studies of cells from IL-4−/− mice were conducted in which IL-13 or a neutralizing Ab to IL-13 was added to the priming cultures. CD4+/LECAM-1high T cells from IL-4−/− mice primed under baseline conditions and restimulated produced 386 pg/ml of TGF-β. Addition of IL-13 or anti-IL-13 to priming cultures induced 340 pg/ml and 396 pg/ml of TGF-β, respectively, following restimulation. Based on these data, it is unlikely that IL-13 regulates TGF-β production in these mice.

Table VI. *The presence of TGF-β in priming cultures enhances the amount of TGF-β produced following secondary restimulation*[a]

Priming Conditions	Secondary Stimulation Conditions: $CD4^+$ T Cells + PB-anti-CD3 + anti-CD28 + IL-2		
	TGF-β (pg/ml)	IFN-γ (ng/ml)	IL-4 (U/ml)
Expt. 1			
$CD4^+$/LECAM-1^{high} T cells + TDS + OVAp + IL-2	801	2.8	1,424
+IL-4	1,227	<0.3	112,158
+TGF-β	1,322	<0.3	646
Expt. 2			
$CD4^+$/LECAM-1^{high} T cells + TDS + CYTO-Cp + IL-2	150	1.8	<10
+TGF-β (5 ng/ml)	770	ND	<10
+TGF-β (1 ng/ml)	550	ND	<10
Expt. 3			
$CD4^+$/LECAM-1^{high} T cells + TDS + CYTO-Cp + IL-2	180	<0.3	<10
+TGF-β	393	<0.3	<10
+anti-TGF-β	225	<0.3	94
+IL-4 (1000 U/ml)	412	<0.3	12,390
+IL-4 (1000) + TGF-β	419	<0.3	<10
Expt. 4			
$CD4^+$/LECAM-1^{high} T cells + TDS + CYTO-Cp + IL-2	350	<0.3	99
+TGF-β	822	<0.3	43
+anti-TGF-β	404	<0.3	230
+IL-12	239	44.9	<10
+IL-12 + TGF-β	655	6.5	<10

[a] Sorted $CD4^+$/LECAM-1^{high} transgenic T cells (5×10^5) from cytochrome *c* TCR/Tg or OVA-TCR/Tg were stimulated for 4 days with TDS (3×10^6), peptide (1 μM), or IL-2 (10 U/ml) in the presence of anti-IL-4 (20 μg/ml), IL-12 (1 ng/ml), IL-4 (1000 U/ml), TGF-β-1 (5 ng/ml or 1 ng/ml), or anti-human TGF-β-1 (10 μg/ml). Cells were washed extensively, recultured in serum-free media, and restimulated at 2×10^5 cells/200 μl on plates coated with anti-CD3 (PB-anti-CD3)$^+$ soluble anti-$CD28^+$ IL-2 for 36 h. Supernatants were harvested and TGF-β, IL-4, and IFN-γ content was measured by ELISA. Values represent the means of triplicate cultures. The SEM was <10% for all cytokines.

The presence of TGF-β in priming cultures has a positive effect on the generation of TGF-β-producing cells

Recognizing that cytokines, e.g., IL-4, play an important role in their own regulation, we next studied the effect that TGF-β itself had on the induction of TGF-β-producing cells. As shown in Table VI, stimulation of naive $CD4^+$ T cells in priming cultures in the presence of TGF-β (using T cells derived from either OVA (Expt. 1) or CYTO-C-TCR/Tg (Expts. 2–4) mice) led to enhanced TGF-β production in secondary cultures. These data are consistent with two potential mechanisms of such self induction: 1) a direct effect of TGF-β on T cells; and 2) an indirect effect mediated by the ability of TGF-β to inhibit IFN-γ production. Evidence in support of an indirect effect through inhibition of IFN-γ is shown in experiment No. 1 using $CD4^+$ T cells from OVA-TCR/Tg, in which the presence of TGF-β in priming cultures and enhancement of TGF-β production in secondary cultures was associated with complete inhibition of IFN-γ production in secondary cultures but no increase in the production of IL-4. In addition, in experiment No. 4, partial reversal of the enhancement of TGF-β priming by the presence of TGF-β in the primary cultures was seen by the addition of IL-12 to the priming cultures, which was associated with increased IFN-γ production. Evidence in support of a direct effect of TGF-β on TGF-β induction is shown in experiments No. 2 and 4 using $CD4^+$ T cells from CYTO-C-TCR/Tg mice. In these studies, addition of TGF-β to priming cultures resulted in a two- to fivefold increase in TGF-β production in the absence of detectable IFN-γ production. Thus, TGF-β is able to positively regulate its own induction.

We also evaluated the effect that TGF-β had on priming for TGF-β, IL-4, and IFN-γ production when added to priming cultures in the presence of IL-4 or IL-12. The addition of TGF-β to priming cultures in which IL-4 was also added (experiment #3) abrogated the increase in IL-4 production normally seen with priming in the presence of IL-4 alone but maintained priming for TGF-β production. Furthermore, the addition of TGF-β to priming cultures in which IL-12 was also added abrogated most of the increase in IFN-γ production following restimulation caused by IL-12. Thus, the presence of exogenous TGF-β in priming cultures appears to have a negative regulatory influence on IL-12 and IL-4 priming for IFN-γ and IL-4, respectively, but still augments the amount of TGF-β produced in secondary cultures. Finally, it should be noted that in all of the studies reported here, priming cultures were done in complete media containing 10% FCS that itself contained approximately 100 pg/ml of TGF-β by ELISA (data not shown). Since addition of anti-TGF-β did not alter production of any of the cytokines tested (Table VI), it is unlikely that the levels of endogenous TGF-β present in the cultures influences priming for TGF-β.

Discussion

The approach to the study of the regulation of TGF-β production adopted here is that used previously to characterize the conditions necessary for priming naive $CD4^+$ T cells for Th1 or Th2 differentiation (i.e., IFN-γ or IL-4 production, respectively). Using this system, we first showed that $CD4^+$/LECAM-1^{high} T cells (naive) did not produce detectable TGF-β or IL-4 36 h after primary stimulation under baseline conditions. This indicated that naive T cells were a suitable starting point for evaluating factors regulating the differentiation of TGF-β-producing T cells. By contrast, $CD4^+$/LECAM-1^{low} (memory) T cells produced measurable, albeit low, amounts of TGF-β under baseline conditions; the level of TGF-β produced by these cells was not appreciably influenced by the presence or absence of IL-4 or IFN-γ, cytokines shown to influence TGF-β production of naive T cells (see below). Thus, $CD4^+$ T cells expressing a marker consistent with previous activation appear to be relatively resistant to further regulation following short-term in vitro stimulation. It should be noted, however, that such nascent memory cells may later regain some ability to be

regulated, since LECAM-1^{low} (memory) cells isolated from mouse lymphoid cells and then primed for several days and restimulated produced variable amounts of TGF-β, depending on the conditions present in the priming culture (our unpublished observations).

In further studies, we showed that two somewhat interrelated priming conditions favor the differentiation of naive CD4+ T cells into cells producing TGF-β: one was the presence of IL-4 and the second was the presence of the Th1 cytokine antagonists, anti-IL-12 plus anti-IFN-γ Abs, in the priming culture. This was seen most dramatically in cultures of naive TCR/Tg CD4+ T cells derived from a strain of mice (B10.A) that produces relatively low amounts of TGF-β under baseline conditions but was also seen with similarly stimulated cells derived from a strain of mice (BALB/c) that produces relatively high amounts of TGF-β under baseline conditions. Moreover, this result was supported by the fact that the emergence of TGF-β-producing cells in secondary cultures correlated with the induction of cells producing low amounts of IFN-γ and high amounts of IL-4.

Additional support for the importance of low IFN-γ and high IL-4 during priming for induction of TGF-β-producing cells was obtained from studies of IFN-$\gamma^{-/-}$ and IL-$4^{-/-}$ mice. The most informative finding in these studies was that total/unsorted CD4+ T cells (containing both LECAM-1^{high} (naive) cells and LECAM-1^{low} (memory) cells) of IFN-$\gamma^{-/-}$ mice produced far higher levels of TGF-β after priming under baseline conditions than did similar cells of IFN-$\gamma^{+/+}$ controls; this correlated with the lack of IFN-γ in cultures containing cells from the IFN-$\gamma^{-/-}$ mice. Conversely, total/unsorted CD4+ T cells of IL-$4^{-/-}$ mice produced far lower levels of TGF-β after priming under baseline conditions than did similar cells of IL-$4^{+/+}$ control mice. This correlated with the high levels of IFN-γ and lack of IL-4 in the cultures containing cells from the IL-$4^{-/-}$ mice. Finally, the presence of IL-10 in priming cultures leading to increased TGF-β production in secondary cultures was associated with decreased IFN-γ. This ability of IL-10 to regulate TGF-β may help explain why IL-$10^{-/-}$ mice have a propensity to develop inflammatory colitis (27). Taken together, these studies provide strong support for the view that the generation of cells producing high amounts of TGF-β occurs under conditions that are similar to those required for the induction of Th2 cells, namely low amounts of IL-12 and IFN-γ and high amounts of IL-4.

Based on the conclusions above, one major mechanism by which IL-4 and IL-12/IFN-γ reciprocally regulate the differentiation of TGF-β-producing cells is via the capacity of these cytokines to cross-regulate one another. This, however, should not obscure the fact that the data obtained show that both IL-4 and IFN-γ have direct (opposing) effects on TGF-β production. The evidence for a direct effect of IL-4 is supported by preliminary studies that show that highly purified naive CD4+ T cells primed with PB-anti-CD3 in the absence of APCs (and thus in the absence of cells capable of producing IL-12) differentiated into cells producing TGF-β, provided that IL-4 was added to the culture (data not shown). In addition, as shown here in experiments with cells from IFN-$\gamma^{-/-}$ mice, the presence of anti-IL-4 in the priming cultures diminished the production of TGF-β in secondary cultures, and the presence of IL-4 in the priming cultures augmented the production of TGF-β in the secondary cultures, demonstrating that IL-4 has a direct effect on TGF-β production. With regard to the mechanisms by which IL-4 can directly influence TGF-β production, two possibilities should be considered. One is that IL-4 has a specific effect on the differentiation of nascent TGF-β-producing cells, and another is that IL-4 enhances TGF-β production in cells already committed to producing TGF-β. Some evidence for the latter possibility is inherent in the fact that the TGF-β promoter contains a potential STAT 6 binding site, and STAT 6 has been specifically linked to IL-4 signaling functions. There is also evidence to show that IFN-γ is an independent regulator of TGF-β production. This includes the fact that the generation of TGF-β-producing cells occurs in the complete absence of IL-4 (i.e., IL-$4^{-/-}$ mice) and that addition of anti-IFN-γ plus anti-IL-12 to priming cultures of total/unsorted cells from IL-$4^{-/-}$ mice caused an increase in TGF-β production in secondary cultures. In addition, cells from IFN-$\gamma^{-/-}$ mice produced more TGF-β than did cells from IFN-$\gamma^{+/+}$ mice, even if the latter's production of IL-4 was blocked by the presence of anti-IL-4 in the priming culture. In considering all these data, we would conclude that the regulation of the differentiation of TGF-β-producing cells with regard to IL-4 and IFN-γ consists of at least three components: 1) a direct positive effect of IL-4 that is important for optimal generation of TGF-β-producing cells; 2) an interactive effect of IFN-γ and IL-4 whereby IL-4 down-regulates IFN-γ production and responsiveness to IL-12 or IL-4 enhances IL-10 production, leading to increased production of TGF-β; and 3 a direct negative effect of IFN-γ that ensures that, in low IFN-γ/IL-12 environments, TGF-β can be produced regardless of the presence of IL-4.

A final issue of interest is the role of TGF-β itself in the regulation of Th1/Th2 responses, as well as it role in its own induction. With regard to TGF-β regulation of Th1/Th2 responses, previous in vitro and in vivo studies have yielded contrasting results. Several reports have shown that TGF-β enhanced the generation of Th1 cells in vitro (28, 29); however, recent work by Schmitt et al. (30) showed that TGF-β can promote or inhibit Th1 development depending on the mouse strain used and the amount of IL-2 present in the priming culture (31). In vivo studies are also discordant as to the role of TGF-β in regulating Th1/Th2 responses. In experimental models of toxoplasmosis or leishmanial infection, TGF-β administration clearly inhibited the generation of a Th1 response (1, 2). By contrast, TGF-β administration to mice infected with *Candida albicans* delayed progression of disease concomitant with lower levels of IL-4 but not of IFN-γ (32). In the present studies, we also examined the role of both endogenously produced and exogenously added TGF-β in the priming of naive CD4+ T cells for IL-4 and IFN-γ production as well as for TGF-β production. We found that addition of TGF-β to priming cultures containing IL-12 markedly reduced the induction of IFN-γ production; it should be noted, however, that this inhibition was not Th1 specific, since the addition of TGF-β also suppressed the generation of IL-4-producing cells. In addition, in Table III*B*, the presence of TGF-β in priming cultures markedly enhanced induction of TGF-β-producing cells but did not appreciably alter production of IL-10 following restimulation (data not shown). Thus, the addition of TGF-β to priming cultures has a direct positive effect on the generation of TGF-β-producing cells as well as an indirect effect mediated by the ability of TGF-β to inhibit IFN-γ production. This ability of TGF-β to positively regulate its own production is consistent with several previous reports showing that TGF-β1 enhanced mRNA expression from a variety of transformed cell lines (33–35); however, while these data provided evidence for autoregulation of TGF-β expression by TGF-β1, they were not done using normal primary CD4+ T cells. In addition, further studies demonstrated that mRNA expression of TGF-β and secretion of protein may not be correlative (36). Thus, our studies provide the first evidence that TGF-β may be the central mechanism controlling TGF-β production from naive CD4+ T cells. Furthermore, as alluded to above, the presence of high amounts of IL-4 and low amounts of IFN-γ may set up the conditions for initial TGF-β production. Then TGF-β, via its production, establishes a positive

feedback loop that can further enhance the magnitude of its production.

One possible caveat to the above conclusions is that the increases in TGF-β production under the various priming conditions studied were on the order of two- to fivefold, which are substantially lower than the concomitant changes in IL-4 and IFN-γ production. In addition, the absolute amount of TGF-β produced by T cells is lower compared with the amounts generated of the other cytokines. It should be pointed out, however, that in an in vivo model of inflammatory colitis, two- to fourfold differences in TGF-β production had a clear effect on suppressing colitis following oral administration of Ag (7). Furthermore, abrogation of responses of this magnitude by administration of anti-TGF-β led to reestablishment of disease. Finally, while $CD4^+$ T cells may not necessarily be the major source of TGF-β in vivo, it is clear from studies of Powrie et al. that transfer of purified $CD4^+$ T cells is sufficient to inhibit colitis in a TGF-β-dependent manner (17). On the basis of these considerations, it seems likely that the observations on TGF-β priming reported here are physiologically relevant. One possibility not addressed in these studies is that IL-4 and IFN-γ also regulate TGF-β production by non-T cells. This could occur, since, as shown in these studies, IL-4 and IFN-γ regulate production of TGF-β, and TGF-β has its own independent effects on TGF-β production even in non-T cells (33–35). Thus, even if a non-T cell lacks receptors for IL-4 or IFN-γ, these cytokines could effect TGF-β production indirectly by TGF-β itself.

To conclude, the studies reported here, defining the optimal conditions for the generation of TGF-β-producing $CD4^+$ T cells, may have important implications for the treatment of autoimmune diseases, especially those diseases mediated by excessive Th1 responses. Thus, since the inhibition of IL-12/IFN-γ production can lead to enhanced TGF-β and IL-4 production, short-term administration of anti-IL-12 (or another IL-12 inhibitor) at the time of specific antigenic challenge may be an effective way to direct a specific T cell response away from Th1 differentiation without at the same time causing long-term nonspecific immunosuppression that would impair the generation of a necessary Th1 response to an intracellular pathogen. A response directed in this way could be particularly useful in the amelioration of autoimmune disease, since it has already been shown that, in experimental models of inflammatory bowel disease (37) or experimental autoimmune encephalomyelitis (38), administration of anti-IL-12 at the time of disease induction or even after the disease is established leads to abrogation of disease progression or even to resolution of disease. While it is believed that the chief mechanism of such resolution is the inhibition of the underlying Th1 response, it is important to emphasize that the same anti-IL-12 treatment also leads to the generation of TGF-β-producing cells, and thus it is possible that the disease is also being affected by the secretion of a suppressor cytokine. Finally, the demonstration that the outcome of oral Ag administration may be manipulated by coadministration of a cytokine antagonist (anti-IL-12) (18), coupled with the possibility derived from the studies reported here as well as recently conducted studies by Weiner and his colleagues (unpublished observations) demonstrating that oral tolerance and TGF-β production are enhanced by coadministration of IL-4, suggests that oral tolerance may indeed be manipulated to provide more effective therapy for autoimmune diseases.

Acknowledgments

We thank Brenda Rae Marshall for editorial assistance.

References

1. Barral-Netto, M., A. Barral, C. E. Brownell, Y. A. Skeiky, L. R. Ellingsworth, D. R. Twardzik, and S. G. Reed. 1992. Transforming growth factor-β in leishmanial infection: a parasite escape mechanism. *Science 257:545.*
2. Hunter, C. A., L. Bermudez, H. Beernink, W. Waegell, and J. S. Remington. 1995. Transforming growth factor-β inhibits interleukin-12-induced production of interferon-γ by natural killer cells: a role for transforming growth factor-β in the regulation of T cell-independent resistance to *Toxoplasma gondii. Eur. J. Immunol. 25:994.*
3. Fukaura, H., S. C. Kent, M. J. Pietrusewicz, S. J. Khoury, H. L. Weiner, and D. A. Hafler. 1996. Induction of circulating myelin basic protein and proteolipid protein-specific transforming growth factor-β1-secreting Th3 T cells by oral administration of myelin in multiple sclerosis patients. *J. Clin. Invest. 98:70.*
4. Karpus, W. J., and R. H. Swanborg. 1991. $CD4^+$ suppressor cells inhibit the function of effector cells of experimental autoimmune encephalomyelitis through a mechanism involving transforming growth factor-β. *J. Immunol. 146:1163.*
5. Miller, A., O. Lider, A. B. Roberts, M. B. Sporn, and H. L. Weiner. 1992. Suppressor T cells generated by oral tolerization to myelin basic protein suppress both in vitro and in vivo immune responses by the release of transforming growth factor β after antigen-specific triggering. *Proc. Natl. Acad. Sci. USA 89:421.*
6. Santambrogio, L., G. M. Hochwald, B. Saxena, C.-H. Leu, J. E. Martz, J. A. Carlino, N. H. Ruddle, M. A. Palladino, L. I. Gold, and G. J. Thorbecke. 1993. Studies on the mechanisms by which transforming growth factor-β (TGF-β) protects against allergic encephalomyelitis. *J. Immunol. 151:1116.*
7. Neurath, M. F., I. Fuss, B. L. Kelsall, D. H. Presky, W. Waegell, and W. Strober. 1996. Experimental granulomatous colitis in mice is abrogated by induction of TGF-β mediated oral tolerance. *J. Exp. Med. 183:2605.*
8. LeGros, G. G., S. S. Ben-Sasson, R. Seder, F. D. Finkelman, and W. E. Paul. 1990. Generation of interleukin 4 (IL-4)-producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4-producing cells. *J. Exp. Med. 172:920.*
9. Swain, S. L., A. D. Weinberg, M. English, and G. Huston. 1990. IL-4 directs the development of Th2-like helper effectors. *J. Immunol. 145:3796.*
10. Seder, R. A., R. Gazzinelli, A. Sher, and W. E. Paul. 1993. Interleukin 12 acts directly on $CD4^+$ T cells to enhance priming for interferon γ production and diminishes interleukin 4 inhibition of such priming. *Proc. Natl. Acad. Sci. USA 290:10188.*
11. Seder, R. A., W. E. Paul, M. M. Davis, and B. Fazekas de St. Groth. 1992. The presence of interleukin 4 during in vivo priming determines the lymphokine-producing potential of $CD4^+$ T cells from T cell receptor transgenic mice. *J. Exp. Med. 176:1091.*
12. Hsieh, C.-S., S. E. Macatonia, A. O'Garra, and K. Murphy. 1995. T cell genetic background determines default T helper phenotype development in vitro. *J. Exp. Med. 181:713.*
13. Schmitt, E., P. Hoehn, T. Germann, and E. Rüde. 1994. Differential effects of IL-12 on the development of naive mouse $CD4^+$ T cells. *Eur. J. Immunol. 24: 343.*
14. Khoury, S. J., W. W. Hancock, and H. L. Weiner. 1992. Oral tolerance to myelin basic protein and natural recovery from experimental autoimmune encephalomyelitis are associated with down-regulation of inflammatory cytokines and differential up-regulation of transforming growth factor β, interleukin 4, and prostaglandin E expression in the brain. *J. Exp. Med. 176:1355.*
15. Chen, Y., V. K. Kuchroo, J.-I. Inobe, D. A. Hafler, and H. L. Weiner. 1994. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalitis. *Science 265:1237.*
16. Weiner, H. L., A. Friedman, A. Miller, S. J. Khoury, A. Al-Sabbagh, L. Santos, M. Sayegh, R. B. Nussenblatt, D. E. Trentham, and D. A. Hafler. 1994. Oral tolerance: immunologic mechanisms and treatment of animal and human organ-specific autoimmune diseases by oral administration of autoantigens. *Annu. Rev. Immunol. 12:809.*
17. Powrie, F., J. Carlino, M. W. Leach, S. Mauze, and R. L. Coffman. 1996. A critical role for transforming growth factor-β but not interleukin 4 in the suppression of T helper type 1-mediated colitis by $CD45RB^{low}$ $CD4^+$ T cells. *J. Exp. Med. 183:2669.*
18. Marth, T., W. Strober, and B. L. Kelsall. 1996. High dose oral tolerance in ovalbumin TCR-transgenic mice: systemic neutralization of IL-12 augments TGF-β secretion and T cell apoptosis. *J. Immunol. 157:2348.*
19. Hsieh, C.-S., S. E. Macatonia, C. S. Tripp, S. F. Wolf, A. O'Garra, and K. M. Murphy. 1993. Development of Th1 $CD4^+$ T cells through IL-12 produced by *Listeria*-induced macrophages. *Science 260:547.*
20. Noben-Trauth, N., G. Kohler, K. Burki, and B. Ledermann. 1996. Efficient targeting of the IL-4 gene in a BALB/c embryonic stem cell line. *Transgenic Res. 5:487.*
21. Dalton, D. K., S. Pitts-Meek, S. Keshav, I. S. Figari, A. Bradley, and T. A. Stewart. 1993. Multiple defects of immune cell function in mice with disrupted interferon-γ genes. *Science 259:1739.*
22. Ohara, J., and W. E. Paul. 1985. B cell stimulatory factor (BSF-1): production of a monoclonal antibody and molecular characterization. *Nature 315:333.*
23. Cherwinski, H., J. Schumacher, K. Brown, and T. Mosmann. 1987. Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. *J. Exp. Med. 166:1229.*
24. Fink, P., W. Gallatin, R. Reichert, E. Butcher, and I. Weissman. 1985. Homing receptor-bearing thymocytes, an immunocompetent cortical subpopulation. *Nature 313:233.*

25. Leo, O., M. Foo, D. Sachs, L. E. Samelson, and J. A. Bluestone. 1987. Identification of a monoclonal antibody specific for a murine T3 polypeptide. *Proc. Natl. Acad. Sci. USA 84:1374.*
26. Groux, H., A. O'Garra, M. Bigler, M. Rouleau, S. Antonenko, J. E. deVries, and M. G. Roncarolo. 1997. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature 389:737.*
27. Strober, W., and R. O. Ehrhardt. 1993. Chronic intestinal inflammation: an unexpected outcome in cytokine and T cell receptor mutant mice. *Cell 75:203.*
28. Swain, S. L., G. Huston, S. Tonkonogy, and A. Weinberg. 1991. Transforming growth factor-β and IL-4 cause helper T cell precursors to develop into distinct effector helper cells that differ in lymphokine secretion pattern and cell surface phenotype. *J. Immunol. 147:2991.*
29. Sad, S., and T. R. Mosmann. 1994. Single IL-2-secreting precursor CD4 T cell can develop into either Th1 or Th2 cytokine secretion phenotype. *J. Immunol. 153:3514.*
30. Schmitt, E., P. Hoehn, C. Huels, S. Goedert, N. Palm, E. Rüde, and T. Germann. 1994. T helper type 1 development of naive CD4+ T cells requires the coordinate action of interleukin-12 and interferon-γ and is inhibited by transforming growth factor-β. *Eur. J. Immunol. 24:793.*
31. Hoehn, P., S. Goedert, T. Germann, S. Koelsch, S. Jin, N. Palm, E. Ruede, and E. Schmitt. 1995. Opposing effects of TGF-β_2 on the Th1 cell development of naive CD4+ T cells isolated from different mouse strains. *J. Immunol. 155:3788.*
32. Spaccapelo, R., L. Romani, L. Tonnetti, E. Cenci, A. Mencacci, G. delSero, R. Tognellini, S. G. Reed, P. Puccetti, and F. Bistroni. 1995. TGF-β is important in determining the in vivo patterns of susceptibility or resistance in mice infected with *Candida albicans. J. Immunol. 155:1349.*
33. vanObberghen-Schilling, E., N. S. Roche, K. C. Flanders, M. B. Sporn, and A. B. Roberts. 1988. Transforming growth factor β1 positively regulates its own expression in normal and transformed cells. *J. Biol. Chem. 263:7741.*
34. Kim, S.-J., K.-T. Jeang, A. B. Glick, M. B. Sporn, and A. B. Roberts. 1989. Promoter sequences of the human transforming growth factor-β1 gene responsive to transforming growth factor-β1 autoinduction. *J. Biol. Chem. 264:7041.*
35. Kim, S.-J., F. Denhez, K. Y. Kim, J. T. Holt, M. B. Sporn, and A. B. Roberts. 1989. Activation of the second promoter of the transforming growth factor-β1 gene by transforming growth factor-β1 and phorbol ester occurs through the same target sequences. *J. Biol. Chem. 264:19373.*
36. Kehrl, J. H., L. M. Wakefield, A. B. Roberts, S. Jakowlew, M. Alvarez-Mon, R. Derynck, M. B. Sporn, and A. S. Fauci. 1986. Production of transforming growth factor β by human T lymphocytes and its potential role in the regulation of T cell growth. *J. Exp. Med. 163:1037.*
37. Neurath, M. F., I. Fuss, B. L. Kelsall, E. Stüber, and W. Strober. 1995. Antibodies to IL-12 abrogate established experimental colitis in mice. *J. Exp. Med. 182: 1281.*
38. Leonard, J. P., K. E. Waldburger, and S. J. Goldman. 1995. Prevention of experimental autoimmune encephalomyelitis by antibodies against interleukin 12. *J. Exp. Med. 181:381.*